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The defining characteristic of a tumor cell is its ability to proliferate under conditions that prevent the growth of a normal cell. Ultimately, cell proliferation is subject to a genetically programmed limit on the number of divisions that a cell can execute. Studies of human and mouse cells in culture have identified two sequential barriers, M1 (cellular senescence) and M2 (crisis/immortalization), which prevent indefinite division.

Circumstantial evidence links the in vitro process of immortalization with the in vivo process of neoplastic transformation. Well characterized tumor suppressor genes must be inactivated as a prerequisite to escape from senescence. Furthermore, escape from crisis is often associated with activation of the telomerase enzyme, a characteristic shared by the majority of human tumors.

Conventional routes have failed to generate a basic understanding of the mechanisms which control cellular mortality. This recommends the use of novel approaches to the problem. I have devised a system, based upon recombinant, replication-deficient retroviruses, that allows a genetic approach to biological problems in animal cells. Application of this system individually to the M1 and M2 mortality controls may allow the identification of genes that enforce finite life-span in vitro and prevent transformation in vivo.

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FOREWORD

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Introduction

Historically, many complex biological problems have surrendered first to attacks that are uniquely available in genetically-tractable organisms. For example, Drosophila provided the first clues to the role of homoedomain proteins in regulating pattern formation during development, and the yeasts played a critical role in unraveling the basic mechanics of cell cycle control. However, many biological questions are best addressed directly in mammalian cells in which, at present, genetic approaches are lacking.

My long-standing interest is in the control of cell proliferation. Specifically, I have focused on the way in which proliferation controls are disrupted when a normal cell is transformed into a tumor cell. In vivo, transformation of a normal cell into a tumor cell may be viewed as a series of genetic changes that result in escape from the intracellular and extracellular constraints which normally regulate cell growth. Normal cells are ultimately limited in the number of divisions which they can perform[6]. In vitro, normal cells can, albeit rarely, be induced to overcome limitations on division potential and become immortal. This immortalization process has emerged as an in vitro model for in vivo neoplastic transformation.

Recent years have seen progress in understanding the mechanisms by which oncogenes and tumor suppressors regulate cell proliferation. However, conventional approaches have substantially failed to provide an entree into those pathways that enforce cellular mortality. This has led me to devise direct, functional approaches through which genes involved in the maintenance of a finite life-span can be sought.

Genetics in animal cells.

Myriad obstacles have hampered the development of a functionally-based cloning approach in animal cells. However, the genetic approach is amply validated by the fact that despite numerous limitations, a few genes have been isolated based solely upon functional criteria (e.g. Tapscott et al., 1988; Schatz et al., 1989; Weinberg et al., 1989; Jung et al., 1995). In each case an extremely powerful selection (i.e. a tight phenotype) was coupled with a cell line that could be

transfected with exceptionally high efficiency. The requirement for these two characteristics has severely limited the range of biological pathways that can be dissected by functionally-based cloning.

To overcome the problem of low gene-transfer efficiencies, investigators have turned to replication deficient retroviruses. These can transduce a wide variety of cell types with efficiencies that approach 100% (Morgenstern et al., 1990). In fact, several recent papers have demonstrated the utility of recombinant retroviruses for functionally-based cloning from cDNA libraries (Whitehead et al., 1995; Rayner and Gonda, 1994; Kitamura et al., 1995). Despite these reports, functionally-based cloning using recombinant retroviruses has failed to gain widespread use. This is likely due to the fact that no one, to date, has presented a coherent system specifically designed for this purpose. Such a system must incorporate several key features. It must deliver efficient gene transfer into a wide variety of cell types, and the drug resistance conferred by a marker gene must coincide with cDNA expression. A simple method should discriminate cells in which a phenotype has been conferred by a virus from variants that occur naturally within the cell population (e.g. phenotypic revertants). Isolation of the viral DNA from infected cells should be trivialized so that either single genes or moderately complex gene libraries can be easily recovered. Genes should be recovered in a form that would simplify a second round of introduction into cells. It is these criteria that have guided my efforts toward the production of a genetic system for use in animal cells (see below).

A genetic approach to cellular mortality control.

In vitro studies have suggested that the mortality of human cells is maintained by two sequential barriers to unlimited proliferation (Shay et al., 1991). These are called M1 (mortality-1) and M2 (mortality-2). When provided with conditions conducive to growth, normal cells (e.g. fibroblasts) are capable of approximately 50-70 population doublings. (Hayflick et al., 1965; Shay et al., 1991). These cells then reach the first genetically programmed proliferation limit, M1. The end result of activation of the M1 control is senescence. (Shay et al., 1991). M1 can be overridden in culture by the expression of viral oncoproteins that inactivate two major

cellular tumor suppressors, Rb and p53 (Shay et al., 1991). Cells which overcome M1 acquire an extended life span, amounting to 20-30 additional divisions. Growth then becomes limited by the second barrier, M2. During M2, also known as crisis, cell proliferation continues but is balanced and eventually overcome by cell death (Shay et al., 1989). Very infrequently, a cell may escape from M2 to yield an immortal clone (Shay et al., 1993).

It has been hypothesized that cells measure their divisional age through changes in telomere length (Levy et al., 1992). In embryonic and germ cells, telomere length is maintained by an enzyme known as telomerase (Kim et al., 1994). Telomerase activity is absent from somatic cells in vivo and from normal cells in culture (Kim et al., 1994). As these cells proliferate, telomeric repeats are lost, due to the inability of the DNA synthetic machinery to completely replicate chromosome ends (Harley et al., 1994). Immortalization is most often accompanied by a stabilization of telomeric repeat length and a re-activation of the telomerase enzyme (Kim et al., 1994). Thus, it has been proposed that M1 is invoked when a subset of telomeres shrinks to a predetermined size (Levy et al., 1992; Harley et al., 1994). This may activate a signaling pathway that results in suppression of cell growth (senescence) through constitutive activation of proteins, pRb and p53, that control the proliferation of a cell during its normal life span. Cells which bypass M1 continue to lose telomere length, possibly until chromosomes become unstable and recombinigenic (Harley et al., 1994). This situation which is probably incompatible with cell survival may constitute M2.

Although the processes of immortalization in vitro and transformation in vivo may not be identical, available evidence points to mechanistic overlap. First, two major tumor suppressor pathways (p53 and Rb) must be inactivated for escape from senescence. Second, immortalization is correlated with a re-activation of the telomerase enzyme. This enzyme is also activated in a majority of human tumors.

The pathways that impose mortality on normal cells are being investigated genetically using the retroviral system described above. To increase the probability of success, M1 and M2 control will be approached individually.

The technical objectives of the proposal were :

1. The availability of a facile system for the isolation of cDNAs by complementation of phenotypes in mammalian cells would allow a fresh approach to a wide variety of biological problems. I propose to develop such a system, based upon recombinant retroviruses, by combining existing, well-established technologies.

2. Limitation of division potential is likely to be one component of tumor suppression in breast and other cancers. The genetic system developed in Aim 1 will be used to search for genes that participate in the maintenance of cellular mortality. Conditionally immortal cell lines in which a temperature sensitive allele or estrogen-dependent of T-antigen has been used to overcome M1 will be used in a screen for cellular genes whose expression can contribute to M1 bypass. This approach may identify regulators of known tumor suppressors, p53 and Rb, that are part of the M1 mechanism. In addition, new insights into previously uncharacterized modes of growth control (e.g. the division clock) might result.

3. The presence of telomerase activity is virtually diagnostic of malignant tumor cells in vivo or of cells that have escaped crisis (M2) and become immortal in vitro (Kim et al., 1995). Activation of telomerase may be a marker for global changes in growth control that accompany transformation or may be the single requirement for immortality. With the goal of understanding how telomerase is reactivated during escape from M2 in vitro and during the formation of breast tumors in vivo, pools of cDNAs will be screened for genes that can induce telomerase activity in normal, telomerase-negative human mammary epithelial cells. This will be accomplished by coupling the retroviral gene transfer system (Aim 1) with a newly developed telomerase assay that can detect as few as 10 telomerase-positive cells.

The elements from the statement of work which were expected to be completed in the the first year of this proposal are as follows :

Statement of work

Technical objective 1 :
Task 1 : Months 1-4 : Development of a retroviral vector for use in the complementation screening system. This task is comprised of the steps necessary to generate the vector shown in Figure 1 of the proposal and to verify its structure by restriction mapping and sequence analysis.

Task 2 : Months 2-8 : Testing of the retroviral vector in model cell lines (e.g. NIH3T3). The completed vector will be tested for the ability to produce high titer

virus upon transfection into retrovirus producer cells. The vector will also be tested for the ability to direct high-level expression of marker genes. The Cre-based recombination system will be used in these model cells to optimize recovery of the integrated provirus from infected cells and for reversion tests in vivo.

Task 3 : Months 7-9 : Preparation of cDNA libraries in the retroviral vectors.

Technical objectives 2 and 3 :
Task 4 : Months 6-9 : Testing of cell lines that will be used in complementation screening for the ability to be infected and for the ability to sustain high level expression of marker genes. For cells that will be used for positive growth selections, this task will also include determination of reversion rates and colony formation efficiencies.

Task 5 : Months 9-24 : Primary screening of cDNA libraries for the ability to induce telomerase in human mammary epithelial cells. This task includes separation of the library into pools containing 1000 cDNA clones each, conversion of these plasmid pools into pools of recombinant viruses, and primary screening of the virus pools.

Progress Report

Progress relating to objective 1 :

The first objective of the proposal was the development of a system that would allow a genetic approach to a wide range of biological problems in animal cells. This objective has been substantially accomplished during the first year of this grant. Progress on individual elements of the system are described separately below :

Retroviral vectors (task 1,2) :

As described in the original proposal, we had concieved a set of retroviral vectors that would allow us to efficiently deliver genes into animal cells (see appendix 1). The unique characteristic of these vectors is that they were designed to be recovered from infected cells via a recombinase-based strategy in a form that could be used directly to generate virus for subsequent cycles of screening/confirmation of phenotype (appendix 2). These vectors have

substantially performed as expected and their ability to enforce the expression of exogenous genes has been confirmed in a wide variety of cell lines/types (e.g. NIH-3T3, Mo7e, Hep3B, 293, rat1, MEF, HMEC, primary uroepithelial cells, NMuMg etc). In each case, gene transfer rates ranged from 10-100%. We have also demonstrated that virus could be recovered from each of these cell types. Using roughly 10,000 cells, several hundred colonies containing excised provirus was recovered from each cell type. This excised provirus could then be transfected into a packaging cell line (see below) and used to produce retrovirus which could infect the original cell type with an efficiency that ranged from 20-50% of that obtained with the intact (e.g. two-LTR) plasmid.

Packaging cells (task 1,3) :

The packaging cell lines that were available at the time of submission of the original proposal had a number of disadvantages. The most severe was that the ability of these cells to produce virus diminished rather rapidly as the cells were passed in culture. In fact, after only two months of passage, Bosc-23 cells were unable to produce measurable titers on NIH-3T3 cells. This forced us to design and produce a new generation of packaging cells which we have called LinX cells. Like Bosc, LinX cells are based upon the 293T cell line. This was chosen for its exceptionally high transfection rates and also because it had given rise in the past to good packaging cells. To address the major drawback of the Bosc cells, we designed a packaging construct that would allow continuous selection for the expression of packaging functions. The gag/pol and envelope cassettes were linked to selectable markers using an IRES (internal ribosome entry site) sequence to produce a bi-cistronic mRNA. Thus, inclusion of the drug to which the selectable marker conferred resistance allowed confirmation of the expression of the mRNA encoding the packaging function. In order to obtain high-level expression of the packaging functions, we chose to deliver packaging functions on a high-copy episomal vector. After experimentation with EBV (epstein-barr virus), we decided to design a new episomal vector system to obtain more consistent and higher expression levels.

This episomal system was based upon bovine papillomaviruses. BPV vectors have been previously used in a number of contexts to obtain high level expression of exogenous genes. The drawback of this system was that episomal replication was limited to a few murine cell lines. Last year, a colleague at Cold Spring Harbor Labs discovered that by providing the E1 and E2 replication proteins in trans, the replication of the BPV origin could be extended to other cell types. We therefore designed a vector system that could be used to provide packaging functions in 293T cells. This vector contains a BPV replication origin, E1 and E2 expression cassettes, sequences necessary for replication and propagation in bacteria, and an expression cassette that provides packaging functions (see appendix 3). Upon transfer into 293T cells, this vector allowed the production of packaging cell lines that were several fold more efficient than any that was available at that time.

Construction/normalization of cDNA libraries (task3):

Once the system had been constructed the next step was to produce cDNA libraries for use in genetic screens. For the purposes of the work described here, libraries were prepared from a transformed, telomerase-positive cell line (HT1080). Libraries have been constructed in our retroviral vectors the complexity of which exceeds 10,000,000 clones. Libraries of this complexity have a 99% chance of containing a cDNA that represents an mRNA that was present at one copy per cell. In order to reduce the number of individual cDNA clones that would have to be screened in order to saturate the library, we have normalized all of the retroviral libraries that we have produced. Normalization is a process in which the relative abundance of cDNAs in a population is equalized to the extent possible using hybridization kinetics. We have modified a procedure originally described by Soares (Soares et al., 1994). This procedure is presented graphically along with some sample analysis of a representative libary in appendix 4. Using our normalization procedure, we routinly reduce the relative abundance of common transcripts such as GAPDH and actin by at least 10-fold while increasing the relative abundance of rare

transcripts such as myc and ras by roughly 5-fold. We estimate that this substantially reduces the number of clones that must be surveyed in any genetic screen.

Validation of the system

One of the most critical aspects of the work that has been done over the first year of this proposal has been to validate both the system and the genetic approach to biological problems in animal cells. One of the assumptions that was made in the original proposal was that one could apply a complementation-screening approach to animal cells. This was, however, only an assumption. Before delving into the rather difficult genetic screens proposed in the original application, it made sense to try to complement phenotypes that were better-understood. It also made sense to chose phenotypes for which selection was not labor-intensive or lengthy as are those described in objectives 2 and 3. Since this work is not directly relevant to the proposal, I will not present it in detail here. Instead, I will summarize our progress one one such genetic screen solely for the purpose of demonstrating that the approach/system has been validated.

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that inhibits the proliferation of many different epithelial cell types. This inhibition correlates with a number of molecular events including activation of the expression of growth inhibitors (e.g. p15INk4b) and repression of growth promoters (e.g. myc). While some progress has been made on deciphering the signalling pathways and cellular responses that lead to growth arrest in the presence of TGF-β, neither the essential downstream targets nor the signal transduction pathways have been well-defined. It was, however, previously established that ectopic expression of myc or of viral oncoproteins that interfere with the Rb pathway (e.g. HPV-16 E7) could bypass arrest. We therefore chose this phenotype as a model for the validation of our genetic system.

We chose to work in Mv1Lu cells both because their response to TGF- β had been well-characterized and because they were easily infected with our retroviruses. Mv1Lu cells were infected with a cDNA library composed of cDNAs from Balb-3T3 cells. 100 individual pools containing ~10,000 infected cells each were passed in the presence of TGF-β for two months. During that period approximately 40 of the plates were lost because no cells could sustain proliferation in the presence of the cytokine. Proviruses were recovered from the remaining 60 plates. In approximately 30 of the 60 plates, one species had been substantially enriched (to >60% of the population). Sequence analysis of the enriched proviruses revealed that cDNAs encoding three different genes had been recovered multiple times. We have initially focused on these three clones.

The first of these clones was myc which was recovered approximately 10 independent times. As mentioned above, ectopic myc expression had been previously shown to bypass TGF-β-mediated arrest. The second clone encoded mdm-2. This pointed to a potential connection between p53 and tgf- β -mediated arrest, since the best-characterized function of mdm-2 is to interfere with p53 function both by masking the p53-activation domain and by promoting p53 degradation. In our hands, however, other methods of interfering with p53 function (e.g. dominant-negative alleles) did not rescue arrest. This suggests that an alternative function of mdm-2 such as its interaction with components of the Rb pathway (Xiao et al., 1995; Martin et. al., 1995) might hold the key to its ability to bypass tgf- β. Finally, we found that ectopic expression of NF-1X allowed growth in the presence of tgf- β. NF-1X is member of the NF-1 family of transcriptional activators that appears to interfere with the ability of NF-1c to activate transcription. NF-1 binding sites have previously been implicated as key elements of tgf- β -responsive promoters. Through this and through other pilot screens, I believe that we have validated the use of our genetic system to find genes that function in specific biological pathways.

Progress related to objective 2 :

Characterization of cells for M1-bypass screens (task 4) :

Our original plan for searching for genes that would allow bypass of the first mortality control (M1) relied on the use of cells which were conditionally immortalized by SV40 large T antigen. These cells would proliferate under conditions in which T-antigen. However, upon inactivation of T-antigen, the cells arrest due to re-assertion of the M1 control. We have tested two cell lines that have been designed in this manner, AR5 (H. Ozer) and IDH4 (Jerry Shay). Initial tests revealed that AR5 was generally too unhealthy to be used in a genetic screen. IDH4 has shown more promise, however, the ability of these cells to tightly arrest is sensitive to the cell density. This emerges as problem in virtually all growth-arrest screens. Infection rates in IDH4 are also rather low (~10%) but are within the range which we consider suitable for screening. Now that we have suffered through the process of learning how to work with these cells, we are currently reconstructing the screen. This involves the delivery of viral oncoproteins which we anticipate should rescue the phenotype (e.g. E6 and E7; SV40 T -- not conditional; etc.). I would anticipate that screening in these cells should be possible within the next few months if no further difficulties are encountered.

As a complement to the use of conditionally immortal cells, we are examining the feasibility of taking a direct approach to the question. Primary human mammary epithelial cells (HMEC) senesce with a fair degree of synchrony around passage 22. These cells can acquire an extended life-span upon introduction of viral oncoproteins, particularly those that interfere with p53 function. Since we can grow and manipulate these cells fairly easily in culture, we plan to introduce cDNA libraries into mid-passage (e.g. p18) HMEC cells and ask whether any infected cells can proliferate significantly past the point where the remainder of the population enters senescence. This is a more time consuming approach than the one described in the original application; however, it has the advantage of addressing the question in the directly relevant

cell type. The likelihood of success in this approach will rest largely upon the rate at which these cells spontaneously escape M1, and this is a parameter that is currently being measured.

Progress related to objective 3 (tasks 4 and 5) :

Objective 3 had as its goal the discovery of genes that regulate telomerase in primary HMEC cells. Much of the characterization described for task 4 has been completed. We are able to grow healthy HMEC populations that retain a telomerase-negative character. We can transfer, using our retroviral system, genes into approximately 50% of the cell population. This gene expression is strong and is maintained indefinitely. Introduction of E6 into our cell populations increases telomerase activity in much the way that has been described (Klingenhutz et al., 1995). As described above the libraries necessary for this screen have been prepared, and once a suitable stock of cells has been obtained, screening should proceed.

Prior to this point, we decided to survey the oncogenes that were available in the lab to ask whether any of these could induce telomerase in HMEC cells. Although cyclin D1, ras, E1A and cdc25 had no effect, preliminary data indicates that introduction of myc, mdm-2 or dominant-negative p53 caused a measurable induction of activity. Of these, the effect with myc was the most striking.

Telomerase was activated by myc to a level that approximates activation by E6. This yields only a fraction of the activity that is seen in a fully transformed cell line; however, partial activation by several different oncogenes raises a number of questions concerning how telomerase is activated as a cell progresses through mortality checkpoints on its way to the transformed phenotype. It has always been assumed that telomerase is either on or off. In essence what has been expected is the existence of a switch that is flipped when a cell achieves immortality. This is consistent with much of the data, but is at odds with the fact that functionally immortal stem cells also posses telomerase activity, but at a level that is much lower than that seen in

transformed cells. This fact indicated that telomerase might be regulated by degrees. It is, in fact, possible that the high level of telomerase activity in transformed cells reflects a series of activating events rather than just a single quantum switch. We are currently testing this hypothesis in several ways. First, we are trying to achieve a quantitative telomerase assay so that degrees of activation can be measured with confidence. Second, we will ask whether the effects of myc plus E6 (for example) are additive. Third we are broadening our survey of oncogenes to identify those which can partially activate the enzyme. These will then be tested to see if their effects are additive. If, in fact, some effects are additive while others are not, then this approach can be used to place different oncogenes in complementation groups and possibly provide clues to the mechanisms by which various oncogenes contribute to enzyme activation.

A telomerase subunit (hEST2) with characteristics of a reverse transcriptase has recently been reported (Nakamura et al., 1997; Meyerson et al., 1997). The expression of this subunit is high in telomerase-positive, immortal cells but is low in telomerase-negative, normal cells. We are currently testing whether expression of this subunit is limiting for activity in HMEC cells and whether expression of any of the genes that affect telomerase activity affect the expression of hEST2.

It is our hope that the preliminary reconstruction experiments that we have performed with oncogenes not only serves to validate the system but might provide insights into the biology of telomerase activation that will allow us to further hone our approach.

Conclusions

We have developed a function-based genetic screening system for animal cells. This system has been validated in a number of screens, and preliminary results suggests that the system will be applicable to a wide range of biological problems. We have begun to develop two screening approaches that address the M1 and M2 mortality controls. As part of the reconstruction

experiments surrounding the M2 screen, we have identified three new inducers of telomerase, myc, mdm-2 and mutant p53. To date, the only known inducer of telomerase was the viral oncoprotein, E6. Our results suggest an alternative framework for thinking about telomerase activation in tumor cells, and we are pursuing this hypothesis in addition to pursuing a genetic screen for telomerase-inducers that is free of preconceived notions.

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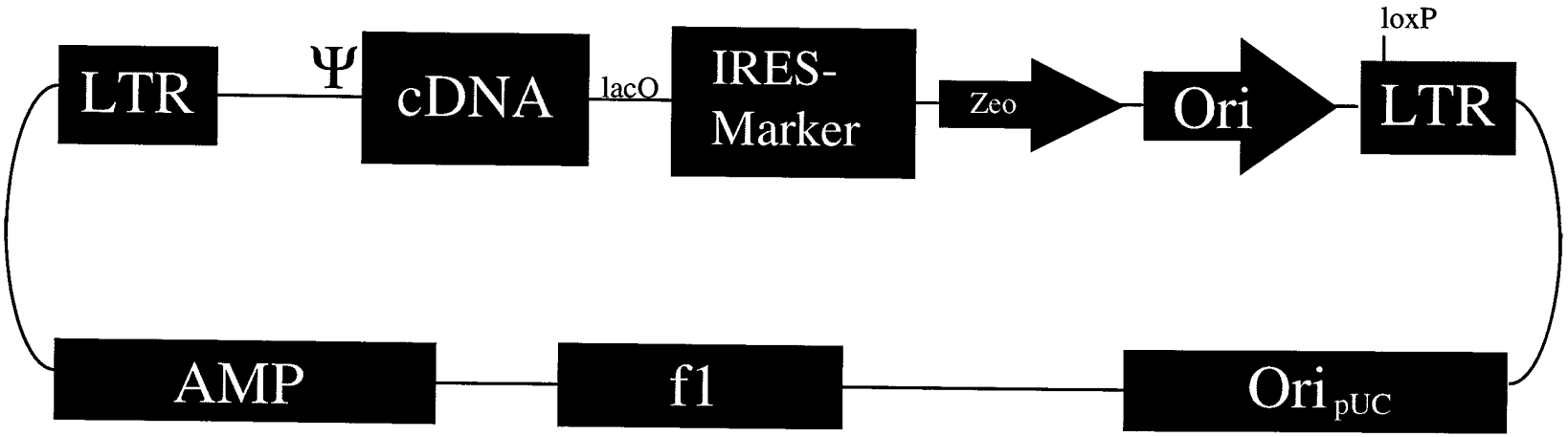
Appendix 1

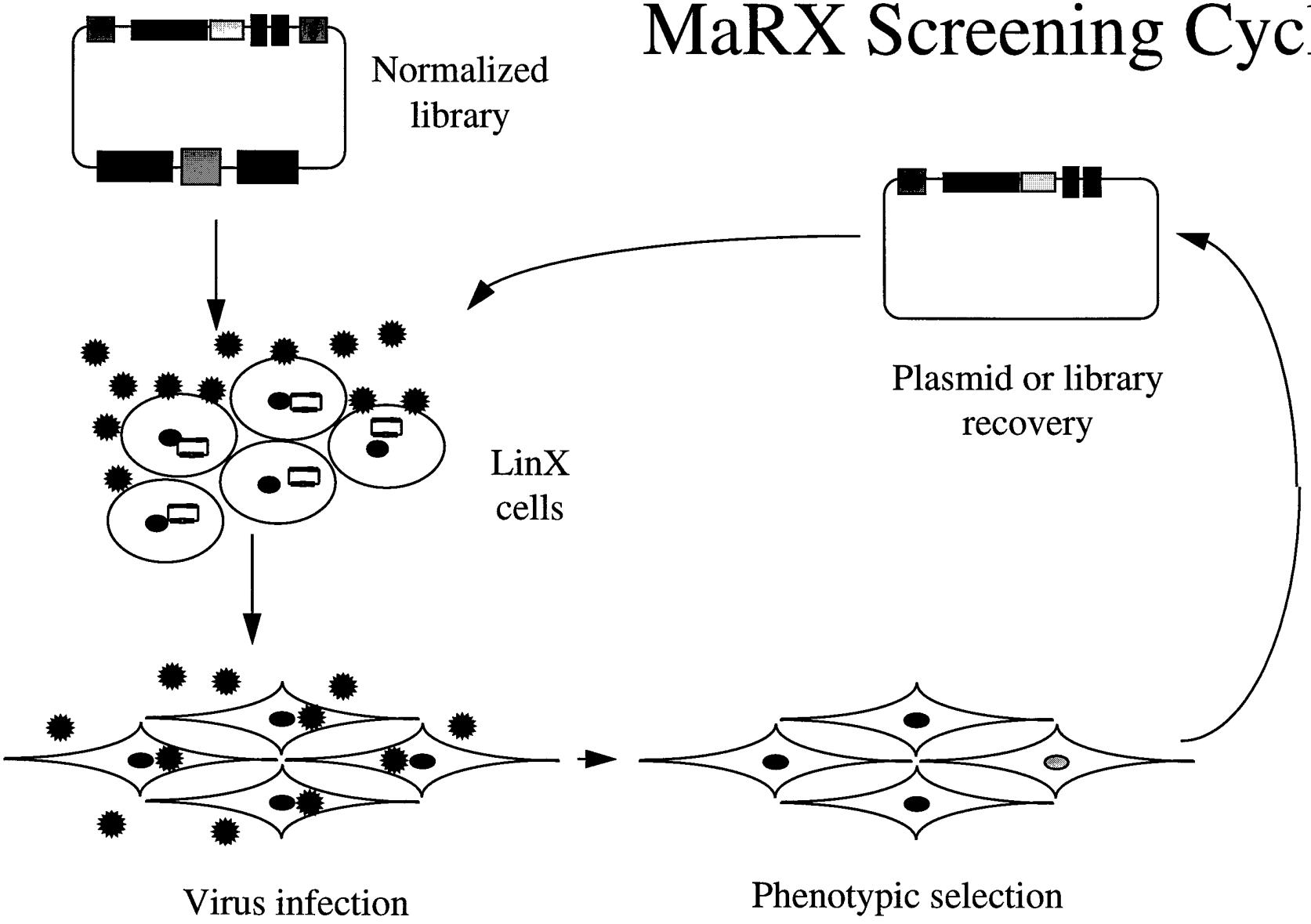
pMaRX II



- Essential elements
  - Promoter -- Ltr, CMV or tetCMV
  - Selection marker -- hygro, puro, neo, zeo, blast
  - Excision element -- loxP, FRT, R
  - Recovery element -- origin, lacO, marker
  - f1 origin

Appendix 2

MaRX Screening Cycle

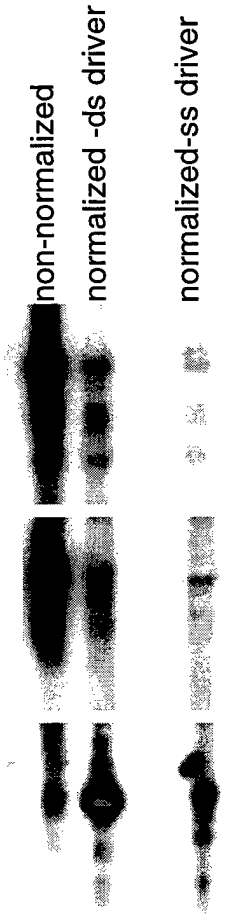


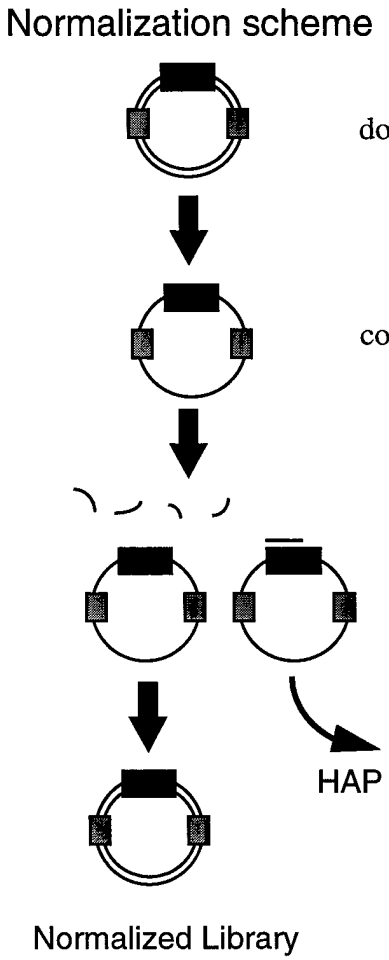
Appendix 3

BPV ori
pUC ori
Amp
BPV-E1
SVpro
BPV-E2
SVpro
bGH pA
ires-hygro
env
gag/pol
CMV pro
ψcIH

Appendix 4

Normalization of retroviral libraries





double-stranded library

convert to single stranded library

hybridize to inserts from the library to C0t of 15

remove all hybridized material